REVIEW ARTICLE

Structure, function and mechanism of exocyclic DNA methyltransferases

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DNA MTases (methyltransferases) catalyse the transfer of methyl groups to DNA from AdoMet (*S*-adenosyl-L-methionine) producing AdoHcy (*S*-adenosyl-L-homocysteine) and methylated DNA. The C^5 and N^4 positions of cytosine and N^6 position of adenine are the target sites for methylation. All three methylation patterns are found in prokaryotes, whereas cytosine at the C^5 position is the only methylation reaction that is known to occur in eukaryotes. In general, MTases are two-domain proteins comprising one large and one small domain with the DNA-binding cleft located at the domain interface. The striking feature of all the structurally characterized DNA MTases is that they share a common core structure referred to as an 'AdoMet-dependent MTase fold'. DNA methylation has been reported to be essential for bacterial virulence, and it has been suggested that DNA adenine MTases (Dams) could be potential targets for both vaccines and antimicrobials. Drugs that block Dam could slow down bacterial growth and therefore drug-design initiatives could result in a whole new generation of antibiotics. The transfer of larger chemical entities in a MTase-catalysed reaction has been reported and this represents an interesting challenge for bio-organic chemists. In general, amino MTases could therefore be used as delivery systems for fluorescent or other reporter groups on to DNA. This is one of the potential applications of DNA MTases towards developing non-radioactive DNA probes and these could have interesting applications in molecular biology. Being nucleotide-sequence-specific, DNA MTases provide excellent model systems for studies on protein–DNA interactions. The focus of this review is on the chemistry, enzymology and structural aspects of exocyclic amino MTases.

Key words: *S*-adenosyl-L-methionine, bacterial virulence, DNA methyltransferase, DNA probe, methylation, restriction endonuclease.

INTRODUCTION

DNA MTases (methyltransferases) catalyse the transfer of methyl groups to DNA from AdoMet (*S*-adenosyl-L-methionine), which is a universal methyl group donor. In prokaryotes, DNA methylation has several important roles in transcription, direction of post-replicative mismatch repair, control of DNA replication, cell-cycle control, bacterial virulence and in distinguishing self and non-self DNA. The distinction of self and non-self DNA is associated with R-M (restriction-modification) systems which function as defence mechanisms against infection of bacteria by bacteriophages [1,2]. So far, over 3700 restriction endonucleases are known and over 1700 different bacterial DNA MTases have been sequenced which recognize and methylate almost 300 different DNA sequences (see http://rebase.neb.com) [3].

DNA MTases are classified into two classes based on the position of the methyl group transfer on bases in DNA: exocyclic amino MTases and endocyclic MTases. The exocyclic amino MTases methylate cytosine at the N^4 position or adenine at the N^6 position, whereas endocyclic MTases methylate the cytosine at the C^5 position which lies within the ring [4–6]. It has been suggested that N^4-cytosine and N^6-adenine MTases may be more closely related to each other than to C^5-cytosine MTases with respect to sequence similarity and conserved motifs [7].

The primary structures of the C^5-cytosine MTases share a set of conserved motifs (I–X) in a constant linear order (Figures 1 and 2) [4,8–11]. Several of these motifs are responsible for three basic functions in the methyl transfer reaction: AdoMet binding, sequence-specific DNA binding and transfer of methyl groups from AdoMet to DNA. A structural comparison of N^6-adenine MTase M.TaqI (TaqI MTase) with the C^5-cytosine MTase M.HhaI showed that the catalytic domains of both the enzymes have a very similar fold [12]. On this basis, Malone et al. [7] performed a structure-guided sequence comparison of N^6-adenine and N^4-cytosine MTases. This analysis revealed that N^4-cytosine, N^6-adenine and C^5-cytosine MTases are closely related to one another. Nine conserved motifs, which correspond to the motifs I–VIII and X found previously in C^5-cytosine MTases, were identified in the exocyclic MTases (Figures 1 and 2). Exocyclic amino MTases are subdivided further into six groups (namely α, β, γ, ζ, δ and ε), according to the possible linear arrangements of three conserved motifs, the AdoMet-binding domain (FXGXG), the TRD (target-recognition domain) and the catalytic domain (DPPY) (Figure 1) [7,11]. The majority of exocyclic amino MTases fall into the α, β and γ subgroups. M.BssHI is the only DNA MTase for which the ζ architecture has been confirmed [11,13].

REACTION MECHANISM

Model studies suggest that aliphatic amines require significant activation for nucleophilic attack on to methylsulfonium compounds [14] and show that the methylation of deoxyadenosine takes place initially at N^1, followed by a Dimroth rearrangement [15].

However, it has generally been assumed that the methylation reaction by exocyclic amino MTases simply involves direct transfer of a methyl group from AdoMet to the N^6 position of adenine residues of DNA with inversion of symmetry in a S_N2 reaction. In order to obtain more insights into the enzymatic adenine methylation, Pogolotti et al. [16] used M.EcoRI and isotopically labelled [6-^{15}N]adenine to determine whether the enzyme transferred the methyl group directly to the N^6 position or via an N^1

Abbreviations used: AdoHcy, *S*-adenosyl-L-homocysteine; AdoMet, *S*-adenosyl-L-methionine; 2-AP, 2-aminopurine; Dam, DNA adenine methyltransferase; MTase, methyltransferase; M.BssHI, etc., BssHI MTase, etc.; R-M, restriction-modification; TRD, target-recognition domain.

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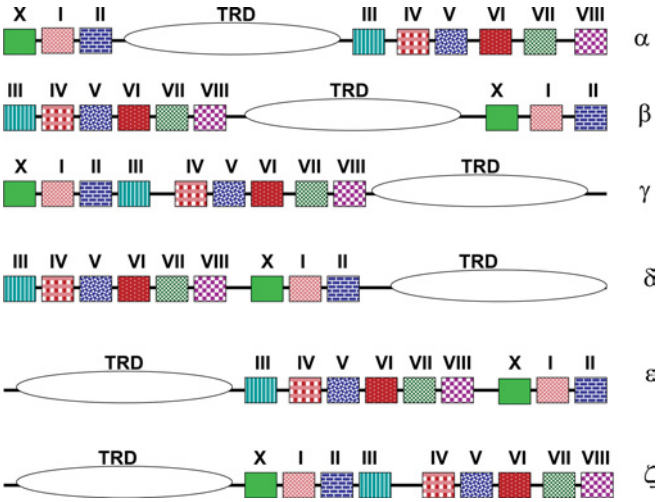

Figure 1 Arrangement of conserved motifs in the primary structure of exocyclic DNA MTases

intermediate, and their results clearly demonstrate that enzymatic adenine methylation takes place directly at the N^6 position. If the methyl group is transferred from AdoMet to the N^6 position of adenine before proton release, a positively charged intermediate (N^6-methylammonium adenine cation) would be formed (Figure 3A), which could be stabilized [17,18]. From the three-dimensional structure of M.TaqI, a N^6-adenine MTase in complex with AdoMet, it has been proposed that the N^6-amino nitrogen of the target adenine is the donor in a hydrogen bond to the side chain of asparagine in motif IV (see below) and possibly to one of the main-chain oxygen of the adjacent two proline residues (motif IV) [19]. This would polarize N^6, activating it for direct transfer of the methyl group from AdoMet. Aromatic amino acids equivalent to Tyr^{108} and Phe^{196} in M.TaqI are present in all DNA and RNA exocyclic MTases [6,7]. The pK_a value of the 6-methylammonium group decreases during and after methyl group transfer, followed by the deprotonation of the 6-methylammonium intermediate. Deprotonation restores the conjugation between the lone pair of N^6 and the adenine ring. The planar 6-methyladenine would sterically overlap with the active-site residues, which would facilitate the movement of methylated adenine back into the DNA helix [20].

On the basis of the structure of the M.PvuII–AdoMet complex and modelled M.PvuII–DNA complex, a catalytic mechanism for N^4-cytosine MTases was proposed (Figure 3B) [21]. In the docking model of M.PvuII, it has been suggested that the N^4 atom of the target cytosine has two possible hydrogen-bond partners: the hydroxy group of Ser^{53} and the main-chain carbonyl of Pro^{54} (the first two amino acids of highly conserved motif IV, SPPF). The phenyl ring of Phe^{56} from the conserved motif IV could make van der Waals contacts with the cytosine rings. Asp^{96} (motif IV) probably forms hydrogen bonds with Ser^{53} and activates its hydroxy group, thereby facilitating the proton transfer from the cytosine amino group through the serine residue and eventually to the aspartic acid residue. As a result of this, the protonated Asp^{96} might form a hydrogen bond with the N^3 of the cytosine. This charge-relay system thus activates the exocyclic amino group of cytosine, which in turn could directly attack the methyl group of AdoMet [21].

KINETIC STUDIES OF EXOCYCLIC DNA MTases

AdoMet-dependent DNA MTases are Bi Bi catalysts, and several kinetic mechanisms can be envisioned for an overall catalytic reaction (Figure 4). The substrate binding may be random (Figure 4A) or ordered (Figures 4B and 4C), and the release of products could be in an ordered or random manner as well. A Ping Pong mechanism or double-displacement mechanism is also possible in which one of the products is formed and released from the enzyme before the binding of other substrate. In this mechanism, the enzyme would be methylated in a first DNA-independent reaction and the methyl group would subsequently

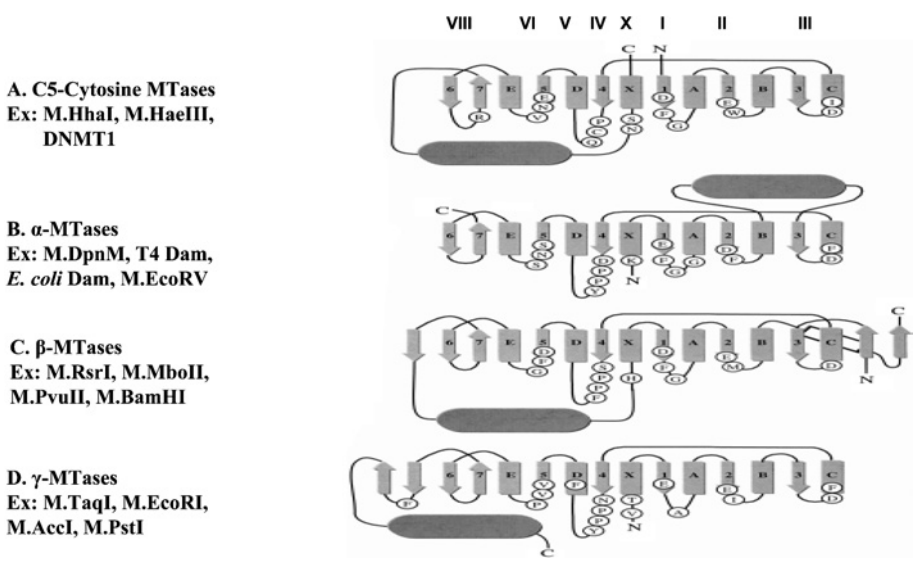

Figure 2 Comparison of the topologies and location of conserved motifs in DNA MTases of different families

Locations of the conserved motifs displayed on the framework of the structure of the large domain of C^5-cytosine MTases. Topology and locations of important amino acid residues in different MTases are shown [5]: C^5-cytosine MTases (**A**), α-class MTases (**B**), β-class MTases (**C**), and γ-class MTases (**D**). Adapted from [5] by permission of Wiley-VCH and the author.

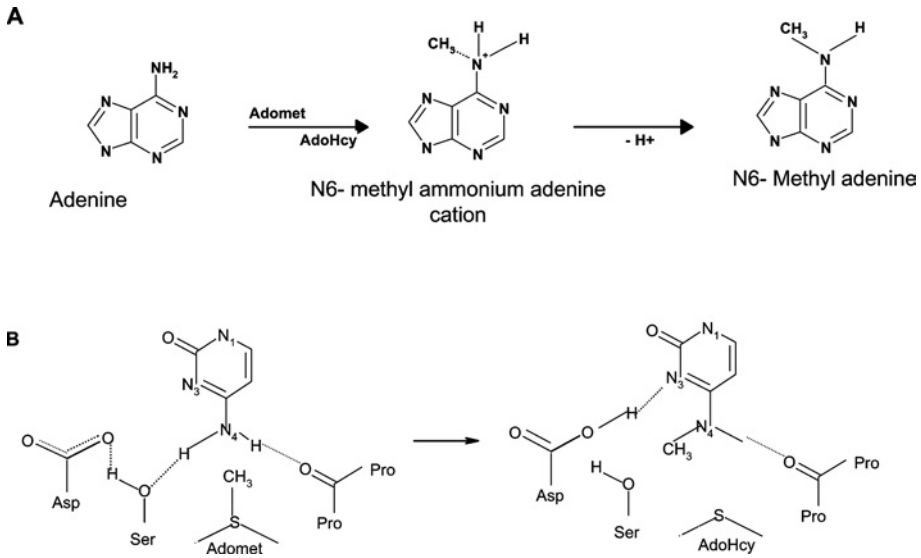

Figure 3 Proposed reaction mechanisms for exocyclic DNA methylation

(**A**) Amino methylation via cationic intermediate [17]. (**B**) Proposed reaction mechanism for N^4-cytosine MTases such as M.PvuII.

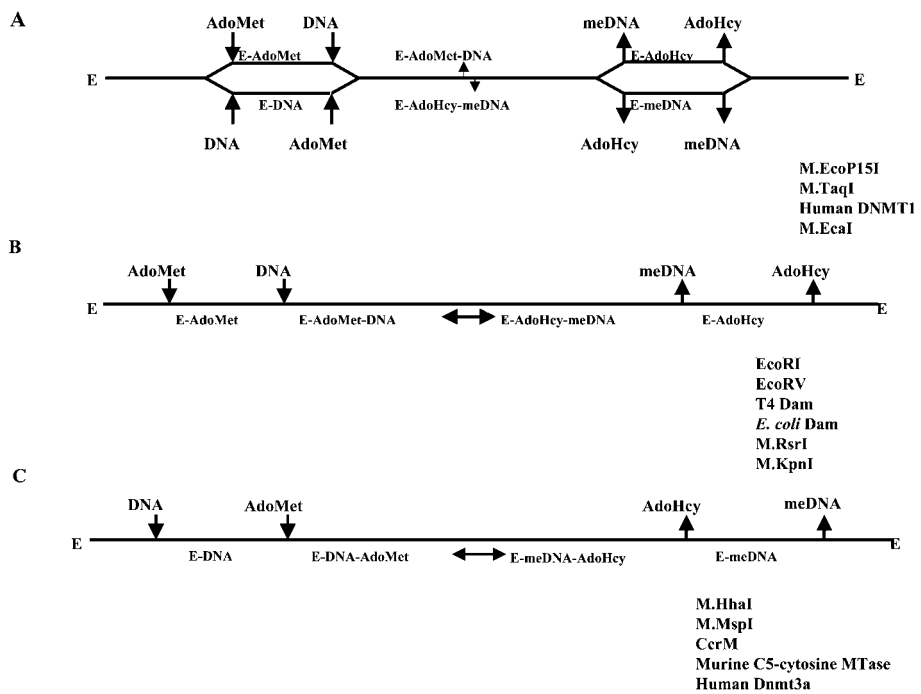

Figure 4 Sequential kinetic mechanisms observed with AdoMet-dependent DNA MTases

(**A**) Random Bi Bi mechanism. (**B**) Ordered mechanism, AdoMet binding first. (**C**) Ordered mechanism, DNA binding first. meDNA, methylated DNA.

be transferred to the DNA in an AdoMet-independent reaction. The sequence of two methyl-transfer events would require stereochemical retentions of configuration at the methyl group. Studies carried out for the steric course of the methyl-transfer reaction using C^5-cytosine MTase M.HhaI and an N^6-adenine MTase M.EcoRI, showed that the methyl-transfer reaction proceeded with an inversion (S_N2 mechanism) of configuration [22]. Therefore these results argue for the absence of a Ping Pong mechanism, but suggest that a sequential mechanism is possible in which a ternary complex of the enzyme is formed. A limited number of DNA MTases have been characterized in terms of kinetic mechanisms. All C^5-cytosine MTases studied so far follow an ordered Bi Bi mechanism with DNA binding first, followed by AdoMet binding (Figure 4C) [5]. The kinetic mechanisms for M.BamHI [23,24], a N^4-cytosine MTase, and M.EcoP15I [25], M.EcoRI [26], M.RsrI [27], M.EcaI [28], CcrM [29], M.TaqI [30], M.EcoRV [31], T4 Dam [32], EcoDam [33,34] and M.KpnI [35], all N^6-adenine MTases, have been elucidated. In general,

Table 1 Kinetic parameters for exocyclic DNA MTases

HM, hemimethylated; L, linear; SC, supercoiled; UM, unmethylated.

Enzyme	k_{cat} (s^{-1})	$K_{m\ (DNA)}$ (M)	$K_{m\ (AdoMet)}$ (M)	$k_{cat}/K_{m\ (DNA)}$ ($M^{-1} \cdot s^{-1}$)	DNA substrate	Reference
M.EcoRV	9.4×10^{-5}	7.1×10^{-6}		0.013×10^{3}	12 UM	[36]
	20.3×10^{-5}	1.0×10^{-6}		0.203×10^{3}	16 UM	[36]
	27×10^{-5}	0.3×10^{-6}		0.9×10^{3}	20 UM	[36]
	16.7×10^{-5}	0.91×10^{-6}		0.184×10^{3}	20 HM	[36]
	44.7×10^{-5}	0.27×10^{-6}		1.66×10^{3}	382 UM	[36]
M.EcaI	0.0047	182×10^{-9}	1.8×10^{-6}	2.58×10^{4}	24 UM	[28,37]
	0.0025	91×10^{-9}		2.7×10^{4}	24 HM	[28]
M.EcoRI	0.11	28×10^{-9}	1.17×10^{-6}	3.8×10^{6}	14 UM	[38]
	0.58	25×10^{-9}		0.23×10^{8}	14 UM	[39]
	0.23	1.3×10^{-9}		1.8×10^{8}	pBR322	[39]
	0.14	24.4×10^{-9}	0.28×10^{-6}	451×10^{6}	14 UM	[40]
	0.105	42.9×10^{-9}	0.248×10^{-6}	2.45×10^{6}	14 HM	[40]
	0.124	2.44×10^{-9}	118×10^{-9}	0.51×10^{8}	14 bp	[26]
	0.142	0.346×10^{-9}	210×10^{-9}	4.1×10^{8}	pBR322	[26]
	0.58	25×10^{-9}		0.24×10^{8}	14 bp	[39]
	0.25	5.9×10^{-9}		0.42×10^{8}	106 bp	[39]
	0.16	2.1×10^{-9}		0.76×10^{8}	222 bp	[39]
	0.25	2.4×10^{-9}		1.0×10^{8}	429 bp	[39]
	0.37	3.7×10^{-9}		1.0×10^{8}	2326 bp	[39]
	0.30	3.2×10^{-9}		0.94×10^{8}	4361 bp	[39]
EcoDam	0.0041	122×10^{-9}		33.7×10^{3}	14 UM	[41]
	0.316	3.6×10^{-9}	12.2×10^{-6}	8.77×10^{7}	ColEI DNA	[42]
	0.0208	25.5×10^{-9}		816×10^{3}	14 bp HM	[41]
	0.93	17.4×10^{-9}	5.6×10^{-6}		20 bp	[34]
T4 Dam	0.015	6.3×10^{-9}		2.38×10^{6}	20 bp UM	[43]
	0.010	6.0×10^{-9}		1.67×10^{6}	20 bp HM	[43]
	0.014	7.7×10^{-9}		1.8×10^{6}	24 bp UM	[44]
	0.14	1.1×10^{-12}	0.1×10^{-6}	1.27×10^{11}	T4 gt^{-} dam DNA	[45]
M.BamHI	0.0175	2.1×10^{-6}		8.3×10^{3}	12 bp	[46]
	0.053	196×10^{-9}	1.22×10^{-6}	2.7×10^{5}	20 bp UM	[23]
M.EcoP15I	0.0053	8×10^{-7}		6.62×10^{3}	31 bp	[47]
	9.6×10^{-5}	8.3×10^{-7}		1.15×10^{2}	pUC 18	[48]
M.TaqI (60 °C)	0.73	0.6×10^{-6}	3.7×10^{-6}	1.21×10^{6}	36 HM	[49]
M.FokI	4.5×10^{-4}	1.6×10^{-7}		2.81×10^{3}	21 UM	[50]
	0.002	1.3×10^{-7}		1.54×10^{4}	21 HM	[50]
M.ClaI	5.5×10^{-5}	1.3×10^{-6}		42.3	12 UM	[51]
CcrM	3.3×10^{-4}				45/50 HM	[29]
M.BcgI	8.3×10^{-6}	3.0×10^{-7}		27.7	λ DNA	[52]
M.MvaI	7.5×10^{-3}	45×10^{-9}		1.67×10^{5}	14 bp	[53]
M.KpnI	3.3×10^{-4}	0.15×10^{-6}		2.2×10^{3}	pUC18 DNA (SC)	[35]
	7.2×10^{-4}	0.09×10^{-6}		8.0×10^{3}	pUC 18 DNA (L)	
	2.2×10^{-3}	1.95×10^{-6}		1.1×10^{3}	UM	
	5.6×10^{-3}	1.42×10^{-6}		4.0×10^{3}	HM	
M.RsrI	1.9×10^{-2}	–	–	–	–	[54]

N^6-adenine MTases exhibit different kinetic mechanisms. Among the exocyclic adenine DNA MTases, an ordered reaction mechanism has been shown for M.EcoRI [26], M.EcoRV [31], T4 Dam [32] and M.KpnI [35], with the enzymes binding to AdoMet first (Figure 4B), whereas, in the case of CcrM [29], an ordered mechanism was postulated with the enzyme binding to DNA first (Figure 4C). A random mechanism of substrate binding has been reported for the EcoP15I MTase [25], M.EcaI [28] and M.BamHI [23,24] (Figure 4A). It is interesting that these MTases, which contain structurally similar domains, have diverse kinetic mechanisms. Table 1 summarizes the kinetic parameters obtained for all the exocyclic amino MTases studied so far.

The discrimination between DNA sequences is the key feature of DNA recognition and has been extensively investigated with DNA MTases. The interaction of the enzyme with DNA is complicated by the requirements for a chemical transformation of the target sequence. This means that enzymes cannot achieve specificity simply by increasing their affinity for cognate DNA sequences, as very tight binding would compromise catalysis. In general, all DNA MTases, including amino MTases, display very slow turnover or rate of methylation (Table 1). The low turnover coupled with their generally strong binding to their target DNA sequence means that k_{cat}/K_m values are high and that the MTases display fair-to-high specificity for methylation of their target sequences. How do DNA MTases discriminate between DNA sequences with such incredible specificity? All DNA MTases studied to date bind their cognate recognition sequences more strongly than to non-specific DNA, and AdoMet as well as its analogues, AdoHcy (*S*-adenosyl-L-homocysteine) and sinefungin increase the affinity of DNA MTases for their substrates and improve selectivity [47,55–60]. Despite the enhanced effectiveness of MTases on long DNA substrates, most DNA-binding studies have been performed using

Table 2 Substrate (DNA and AdoMet)-binding affinities for DNA MTases

K_d values for DNA reported were determined in the presence of cofactor; (−) indicates the binding constants determined in the absence of cofactor (AdoHcy or sinefungin); (+) with cofactor. HM, hemimethyltated; NSP, non-specific DNA; SP, specific DNA; UM, unmethylated.

Enzyme	$K_{d\ (DNA)}$ (M)	$K_{d\ (AdoMet)}$ (M)	DNA substrate	Reference
M.EcoRV	46×10^{-9}		30 UM	[60]
	11×10^{-9}		30 HM	
	143×10^{-9}		30 DM	
M.EcoRI		30×10^{-6}	14 bp	[26]
		10×10^{-6}		[38]
	0.43		14 bp	[39,61]
	0.29		32 bp	
	0.10		101 bp	
	0.07		378 bp	
	0.02		775 bp	
	4.4×10^{-9}		14 UH	[62]
	4.1×10^{-9}		14 HM	
	$(1–4) \times 10^{-6}$		pBR322	[26]
EcoDam	$(10–20) \times 10^{-9}$		14 bp SP	[41]
	$(10–20) \times 10^{-9}$		14 bp NSP	
	$(60–70) \times 10^{-9}$ (+)	6.31×10^{-6}	14 bp SP	
	$(300–400) \times 10^{-9}$ (−)		14 bp SP	[56]
	1.6×10^{-6}		14 bp NSP	
	$(3–4) \times 10^{-6}$ (−)		14 bp NSP	
	119×10^{-9}		20 bp	[34]
	3.4×10^{-6}		20 bp	[63]
T4 Dam	16.5×10^{-9}		20 bp UM	[43]
	8.5×10^{-9}		20 bp HM	
	18×10^{-9} (+)		24 bp	[44]
	54×10^{-9} (−)			
		7.6×10^{-6}	T4 DNA	[45]
	8.57×10^{-9}		14 bp SP	[64]
	3.3×10^{-9}		14 bp NSP	
M.EcoKI	1.6×10^{-9}	3.6×10^{-6}	45 bp UM	[58]
(M_2S_1)	9.1×10^{-9} (−)	2.21×10^{-6}		
	2.36×10^{-9} (−)		21 bp	[65]
	0.6×10^{-9}			
M_1S_1	13.97×10^{-9}			
	14.67×10^{-9} (−)			
M.EcoP15I	1.0×10^{-6}		31 bp	[47]
M.TaqI		2×10^{-6}		[66]
M.RsrI		6.1×10^{-6}	14 HM	[54]
	17 (+)		UM	
	311 (−)		UM	
	6 (+)		HM	
	17 (−)		HM	
	> 800 (±)		NSP	
M.KpnI		4.1×10^{-6}		[35]
M.BamHI	371×10^{-9} (−)	1.22×10^{-9}		[23]
	200×10^{-9} (+)			

short DNA duplexes. Binding affinities for duplexes containing the target site are typically in the range 10^{-8}–10^{-6} M (Table 2). Most studies use naturally occurring highly polymeric DNA as the substrate to determine the kinetic parameters of the reaction. The analysis of such data is complicated by the fact that many MTases are assumed to use the facilitated diffusion of the bound enzyme in a one-dimensional search for its specific recognition site. However, the use of relatively short oligonucleotide duplexes containing the specific recognition site simplifies the experimental conditions and allows one to obtain more accurate data to calculate reaction parameters.

In order to determine the rate-limiting step of the reaction catalysed by DNA MTase, the rate of accumulation of methylated DNA product is estimated using steady-state reaction conditions. When the progress curve of product formation over time is extrapolated back to the *y*-axis (time zero), the intercept is not zero. The non-zero intercept is most simply taken to indicate that the steady-state rate of catalysis is limited overall by a step in the kinetic pathway after the methyl group transfer step. Such bursts have been observed for M.EcoRI [67], M.PvuII [68] and T4 Dam [43], indicating that the release of products is the rate-limiting step in this reaction. In contrast, the rate of methyl group transfer was the rate-limiting step in the M.TaqI methylation reaction [30]. Reactions under single-turnover conditions ($[E] \gg [S]$) are performed in order to determine the rate of methylation ($k_{methylation}$). M.EcoRI catalyses a very rapid methyl group transfer with the $k_{methylation}$ being 300-fold higher than the reaction rate constant (k_{cat}) [67]. On the other hand, the k_{cat} and $k_{methylation}$ values for M.BamHI are 0.053 s^{-1} and 0.07–0.1 s^{-1} respectively [24]. Pre-steady-state kinetic analysis revealed in the case of T4 Dam that the $k_{methylation}$ for unmethylated and hemimethylated substrates was at least 20-fold greater than the overall k_{cat}, indicating that the release of products is the rate-limiting step in the reaction [24].

By using a combination of pre-steady-state kinetics and spectroscopic approaches, it has been possible to put forward a detailed reaction pathway for M.EcoRI. The enzyme binds DNA at a rate of $1.6 \times 10^7\ M^{-1} \cdot s^{-1}$ and then undergoes a slow isomerization to a catalytically competent form (E-DNA$^{s'}$). Catalysis of the methyl group transfer occurs next at a rate of 200 s^{-1} to produce an enzyme–product complex. Enzyme-methylated DNA exists in two forms: (i) a slow dissociation complex (E-DNA^{M*}) which later isomerizes into (ii) the fast dissociation complex (E-DNA^{M**}). The methylated target adenine (DNA^{M*} and DNA^{M**}) remains in an extrahelical position in both these complexes even after its release from the enzyme. Restacking of the target base takes place at different rates: 0.6 s^{-1} and 50 s^{-1} for the fast and slow dissociation complexes respectively. Therefore the release of the methylated DNA appears to be the rate-limiting step [69,70]. In general, for the DNA MTases studied, except in the case of T4 Dam, the release of the methylated product often limits catalysis.

T4 Dam is similar to bacterial Type II DNA MTases with respect to its kinetic parameters, except that it has lower K_m values for substrates and a higher catalytic constant. The relatively high specificity constant of T4 Dam ($k_{cat}/K_m = 2.4 \times 10^6\ M^{-1} \cdot s^{-1}$) indicates that it is one of the most efficient enzymes among several MTases studied so far (Table 1). On comparing the pre-steady-state and single-turnover methylation kinetics of T4 Dam, it was observed that T4 Dam–AdoMet functions as a monomer under steady-state conditions, whereas under single-turnover conditions, a catalytically active complex containing two T4 Dam–AdoMet molecules was observed initially, and two methyl groups were transferred per duplex [71]. It was also observed that preformed T4 Dam–AdoMet complexes were more efficient than preformed T4 Dam–DNA complexes in the first round of catalysis by pre-steady-state kinetics [71]. Based on these results, an ordered Bi Bi mechanism was proposed for T4 Dam where AdoMet bound first followed by DNA. The proposed mechanism was supported further by initial velocity-dependence studies, product-inhibition and substrate-inhibition studies, where AdoMet binds before DNA and methylated DNA is released first followed by AdoHcy [32]. However, the stimulatory effect of high AdoMet concentrations on methylation rate of DNA indicated that the mechanism was more complex. This effect could be explained if T4 Dam were to possess two AdoMet-binding sites. The first is the catalytic site with a high affinity for AdoMet, and the second is an allosteric site with a low affinity for AdoMet. The presence of two AdoMet-binding sites has been suggested for EcoDam and M.PvuII [57,68]. Free T4 Dam randomly interacts with substrates DNA and AdoMet to form a ternary T4 Dam–AdoMet–DNA complex in which T4 Dam undergoes a conformational change to

attain an active form to carry out methylation. After the methyl group transfer from AdoMet to DNA, methylated DNA dissociates rapidly (1.7 s^{-1}) from the complex. In contrast, dissociation of AdoHcy proceeds relatively slowly (0.018 s^{-1}), indicating that release of AdoHcy is a rate-limiting step. The release of AdoHcy from enzyme–AdoHcy complex can occur by two pathways. The first involves release of AdoHcy, while T4 Dam remains in the active conformation and specifically binds to the second AdoMet molecule. Then, the T4 Dam–AdoMet complex binds to DNA for the next round of catalysis. The other proposed mechanism for methylation is where the release of AdoHcy is co-ordinated with the binding of the second AdoMet in a concerted manner, while T4 Dam remains in the active form. The resulting T4 Dam–AdoMet complex then binds to DNA for the next round of methylation. Thus the role of AdoMet in the methylation reaction of T4 Dam goes beyond that of simply serving as a methyl donor. As a consequence of the AdoMet-induced conformational rearrangement and increased specificity, T4 Dam is capable of undergoing a rapid reorientation to the productive strand in an asymmetrically modified recognition site [71]. While similar results were obtained for the M.RsrI [27] and EcoDam [33], Reich and Mashhoon [67], measuring single turnovers in the reaction catalysed by M.EcoRI, showed that there was no specificity in binding orientation, i.e. the enzyme methylated only 50 % of the substrate–hemimethylated duplexes.

Pre-incubation studies carried out by Urig et al. [33] demonstrated that the preformed enzyme–DNA complex was the preferred way of complex assembly than pre-formed enzyme–AdoMet complex, because the rate of methylation was 10-fold higher for the pre-formed enzyme–DNA complex. On the basis of this result only, it was concluded that EcoDam followed an ordered Bi Bi mechanism where DNA binds first, followed by AdoMet. However, Mashhoon et al. [34] have argued that these results provide little evidence for any order of substrate addition. They provide direct evidence for a competent enzyme–AdoMet complex by isotope partitioning experiments. In addition, using steady-state kinetics and product inhibition studies, Mashhoon et al. [34] have shown that EcoDam follows an ordered Bi Bi mechanism where AdoMet binds first followed by DNA.

PROCESSIVE AND DISTRIBUTIVE MODE OF METHYLATION

In vivo, DNA substrates are much longer and contain many methylation sites. Thus *in vitro* methylation of short single-site duplexes does not take into account possible processive behaviour of the DNA MTases, i.e. movement of enzyme along the DNA via one-dimensional (or linear) diffusion and carrying out multiple turnovers on the same substrate molecule. It has been shown that M.EcoRI scans < 400 bp and M.EcoRV scans at least 1500 bp during linear diffusion [31,39,61].

If an MTase is processive, it is expected that two methyl groups will be incorporated into a hemimethylated DNA molecule containing two sites, resulting in methylation of both sites on a single molecule of DNA. An enzyme that uses facilitated diffusion to locate a specific site might reasonably be expected to locate another site on the same molecule by remaining bound and diffusing along the DNA. This would greatly enhance the efficiency of methylation at the second site by the MTase. A defined steady-state rate of enzyme activity will not be observed because the enzyme need not dissociate from the DNA before encountering another methylation site. However, M.EcoRI was found to methylate two sites on a single DNA molecule in a distributive rather than a processive manner, suggesting that the enzyme dissociates from the DNA before release of the reaction product, AdoHcy. It has been demonstrated that M.EcoRI is not processive with substrates in which adjacent sites are only 50 bp apart [39,61]. The distributive mechanism of DNA methylation of M.EcoRV is a direct consequence of its order of substrate binding because AdoHcy cannot leave the ternary enzyme–DNA–AdoHcy complex and AdoMet cannot bind an enzyme–DNA complex [36].

It has been demonstrated that the CcrM acts processively to methylate two sites in a DNA substrate. Because CcrM is processive, AdoHcy has been shown to be released before fully methylated DNA, indicative of a preferred order in product release. The processive nature of CcrM with hemimethylated DNA probably reflects the role of CcrM in remethylation of the newly replicated chromosomes in the predivisional cell [29].

Using steady-state and pre-steady-state analysis, Zinoviev et al. [72] have shown that T4 Dam modifies 40-mer duplexes with two GATC sites in a processive fashion. During processive methylation reaction, T4 Dam rapidly exchanges product AdoHcy for substrate AdoMet without dissociating from the DNA duplex. The processive mechanism is consistent with the ordered Bi Bi mechanism of T4 Dam.

With EcoDam, there have been conflicting results with regard to the enzyme being processive or distributive. Herman and Modrich [42] could not detect processive methylation of DNA. On the other hand, Bergerat et al. [73] found indirect evidence for processive methylation of up to three GATC sites on plasmid DNA substrates. Urig et al. [33] observed that a functional monomeric EcoDam methylates only one strand of the DNA in each binding event. They showed that EcoDam scans 3000 GATC sites per binding event randomly and methylates GATC sites on DNA processively. In contrast, Mashhoon et al. [34] demonstrated that EcoDam methylates adjacent GATC sites in a distributive manner. This finding has some bearing to the *in vivo* situation. For instance, as the authors suggest, the methylation state of two GATC sites in the Pap regulon in part determines the phase variation of pili formation [34]. One possible reason for the different results obtained could be understood in the context of sequences flanking the GATC-recognition sites. Thus it seems plausible that EcoDam is not processive with particular GATC sites. Furthermore, Peterson and Reich [74] have shown that replacement of the poorly methylated GATC sites with the sites that were predicted to be processively methylated resulted in an increase in Dam processivity.

In general, all MTases that are accompanied by a restriction endonuclease show a distributive mechanism of DNA methylation, whereas all solitary MTases methylate DNA in a processive manner. Is there a biological reason for these related enzymes behaving so differently? The distributive mechanism of DNA methylation is a crucial adaptation of DNA MTases in R-M systems to the biological function of these systems which is to cleave phage DNA by the restriction enzyme. It is important that the endonuclease reaches its site on the phage DNA before it is modified. It therefore makes sense that MTases are distributive which considerably lowers down the rate of DNA methylation, whereas endonucleases, in general, are processive in their mode of action.

BASE FLIPPING

Base flipping is a mechanism where a target base within the recognition sequence in DNA is swung completely out of the helix into an extrahelical position [75]. It was first reported when the co-crystal structure of M.HhaI, a C^5-cytosine MTase, bound to DNA in the presence of AdoHcy was determined [76]. The crystal structure of the M.HhaI–DNA–AdoHcy complex revealed that the target base had been rotated 180° out of the DNA helix via the

minor groove into a catalytic domain (motif IV). Base flipping has been proposed as a general means used by MTases for AdoMet-dependent methylation of DNA [75]. 2-AP (2-aminopurine) has been broadly used as a fluorescent probe for detection of the existence of base flipping [75]. Perturbations of 2-AP within a DNA structure can change its fluorescence behaviour. Fluorescence intensity changes caused by the binding of a protein to DNA is indicative of a change in the environment of 2-AP. Binding of M.EcoRI to the DNA containing the 2-AP modification adjacent to the target base resulted in detectable changes in fluorescence emission spectra, indicating that the extrahelical target base was stabilized in a low dielectric environment of the enzyme [77].

Holz et al. [78] have shown that addition of M.TaqI to duplex oligonucleotides containing a 2-AP modification at the target position in the TaqI-recognition sequence resulted in a strongly enhanced 2-AP fluorescence. On the basis of mutational studies and the three-dimensional structure of M.TaqI in complex with the cofactor, and its structural homology with M.HhaI, Tyr^{108} and Phe^{196} have been suggested to interact with the extrahelical adenine [49]. This suggests that Phe^{196} is important for stabilizing the extrahelical target adenine and is probably involved in placing the extrahelical target base in an optimal position for methyl group transfer.

2-AP fluorescence measurements and Rhodamine-coupled fluorescence anisotropy studies have clearly shown that DNA binding and base flipping transitions are nearly concerted by M.EcoRI [69,70,79]. Recently, Hopkins and Reich [80], using FRET (fluorescence resonance energy transfer) analysis, have shown that the conformationally complex processes of protein binding to DNA, sequence-specific recognition, DNA bending and base flipping occur extremely efficiently.

Using the 2-AP fluorescence-based assay, enhancements in fluorescence upon enzyme binding to its canonical sequences have been demonstrated with M.TaqI [78], M.EcoP15I [81], M.EcoRV [31], M.EcoKI [82], M.RsrI [27], T4 Dam [83], M.KpnI [84] and EcoDam [85]. Interestingly, both M.EcoRV [31] and M.EcoP15I [81] induced enhancement in 2-AP fluorescence when 2-AP was placed at a location other than at the target site, but within the recognition sequence. Thus 2-AP can be used as a probe to study other aspects of DNA structure and dynamics that could be altered by protein binding. With M.RsrI [27], M.EcoKI [82] and M.KpnI [84], not only did binding of the enzymes to DNA containing a 2-AP modification at the target adenine show enhancement of fluorescence intensities, but also the emission peaks had a blue shift of approx. 7–10 nm. Bist and Rao [86] demonstrated that magnesium is an essential metal ion in promoting M.EcoP15I methylation activity and that Mg^{2+} ions are required for stabilization of the extrahelical adenine base which is probably flipped out during the methylation reaction.

Liebert et al. [63] have shown that EcoDam flips the target base in a biphasic reaction; flipping the target base was very fast ($k_{flip} > 240\ s^{-1}$), but binding of the flipped base into the active-site pocket of the enzyme was slow ($k = 0.1–2\ s^{-1}$). Base flipping occurs in the absence of AdoMet, but binding of the target base in the active-site pocket requires AdoMet.

Base flipping analysis with non-canonical sites has shown that target recognition occurs before flipping of the base. All of these observations indicate that DNA recognition occurs first, followed by cofactor binding and base flipping [63].

Although base flipping is well characterized in structural terms, the mechanistic details of this process remain elusive. One fundamental question regarding the mechanism of base flipping is whether it is a process that is actively induced by the enzyme, or whether the enzyme merely captures bases that spontaneously flip out of the DNA helix in a 'breathing' process. Studies with wild-type EcoDam and its mutants indicate that the contacts to the target base stabilize the flipped state, suggesting that the enzyme makes use of active and passive means to promote base flipping [63]. Is there a motif involved in base flipping and stabilization? Estabrook et al. [87] identified a motif that includes a positively charged or polar side chain and a hydrophobic residue positioned adjacent to the target DNA base and either the 5′- or 3′-phosphate which appears in co-crystal structures of both M.HhaI, a C^5-cytosine MTase, and in M.TaqI, a N^6-adenine MTase.

STRUCTURE–FUNCTION STUDIES

Motifs

The primary sequences of the C^5-MTases share a set of conserved motifs (I–X) in a constant linear order (Figures 1 and 2) [1,8–10]. Several of these motifs are responsible for three basic functions: (i) AdoMet binding, (ii) sequence-specific DNA binding, and (iii) catalysis of methyl group transfer. Before any of the DNA MTases were characterized structurally, two motifs were assigned functional roles. It was proposed that motif I (FXGXG) was part of the AdoMet-binding site. This was based on the presence of motif I in a wide variety of AdoMet-dependent enzymes, including all DNA, RNA and several protein MTases [1,8,88–90]. In the case of C^5-cytosine MTases (Figure 2A), it was shown that motif IV which possesses an invariant Pro-Cys dipeptide was involved in methyl group transfer [10,91,92]. Mutagenesis of this dipeptide, leading to loss of function in M.EcoRII, M.HhaI and M.HaeIII, was one of the several criteria proposed for the importance of the Pro-Cys dipeptide sequence in motif IV [10,91,92]. The region between motif VIII and motif IX was found to be highly diverse and was termed as a variable region. This region was shown to be involved in target DNA recognition and was aptly termed as TRD [93,94]. Initially only motifs I and IV could be recognized in the primary sequences of N^4-cytosine and N^6-adenine MTases. On the basis of this finding, Malone et al. [7] performed a structure-guided sequence comparison of N^6-adenine and N^4-cytosine DNA MTases. This analysis revealed that the N^4-cytosine, N^6-adenine and C^5-cytosine MTases were closely related to one another. Nine conserved motifs, which correspond to motifs I–VIII and X found previously in C^5-cytosine MTases, were identified in exocyclic MTases. The amino MTases have been subdivided further into three groups, namely α, β and γ, which are characterized by distinct linear orders for the conserved motifs [7]:

group α: N-terminus–AdoMet-binding region–TRD–catalytic region–C-terminus;

group β: N-terminus–catalytic region–TRD–AdoMet-binding region–C-terminus;

group γ: N-terminus–AdoMet-binding region–catalytic region–TRD–C-terminus.

Group α contains N^6-adenine MTases (Figure 2B), group β contains both N^4-cytosine MTases and N^6-adenine MTases (Figure 2C), and group γ consists of M.TaqI and other N^6-adenine MTases (Figure 2D). This grouping does not really demarcate N^4-cytosine from N^6-adenine MTases. The amino MTases belonging to the group γ have a motif order very similar to C^5-cytosine MTases, except that they differ in the position of motif X. A homologue of motif IX in C^5-cytosine MTases could not be identified easily in amino MTases.

A brief description of some of the motifs present in amino MTases is given below. Motif I, which is conserved among all AdoMet-dependent enzymes, accommodates the methionine moiety of AdoMet. It is analogous to the so-called G-loop in NAD^+-binding enzymes with the consensus sequence GXGXXG.

In α or β amino MTases, the consensus sequence contains a strictly conserved phenylalanine residue, whereas, in the γ amino MTases, this amino acid is missing. The role of motif I in some N^6-adenine MTases has been assessed by site-directed mutagenesis. It was shown in M.EcoKI, a member of the Type I R-M systems, that substitution of aspartic acid for the first glycine residue in motif I completely abolished AdoMet binding, but did not alter the DNA-binding properties of the enzyme [95]. Substitution of arginine or serine for the second glycine residue in motif I of M.EcoP15I abolished AdoMet binding, leading to the loss of enzyme activity [96]. When Phe^{39} in motif I of M.EcoRV was replaced with alanine, the mutant enzyme did not bind AdoMet, although DNA binding was not affected [97]. Motifs II and III are less conserved in amino MTases as compared with C^5-cytosine MTases and are also associated with AdoMet binding. It has been proposed that the conserved charged amino acid (aspartic acid/glutamic acid) present in motif II interacts with the ribose hydroxy groups of AdoMet, whereas aspartic acid of motif III hydrogen bonds to adenine N^6 position. The bulky hydrophobic side chains (part of motif III) make the van der Waals contacts with AdoMet's adenine. The backbone amide groups of amino acids in motif III also make hydrogen-bond contacts with N^1 of the adenine moiety of AdoMet. Substitution of alanine for Asp^{58} (motif II) in M.EcoRV resulted in a mutant variant of M.EcoRV which was defective in AdoMet binding [97].

Motif IV, also called the DPPY motif, based on early sequence comparisons is the most significant among the conserved motifs with the consensus sequence (S,N/D)PP(Y/W/F). Both α and β N^6-adenine MTases, with few exceptions, contain the consensus motif DPPY (Figures 1and 2). The γ subgroup of N^6-adenine MTases (M.TaqI) contains a NPPY sequence, whereas N^4-cytosine MTases contain a SPPY sequence. Several groups have performed mutational studies on amino acids in motif IV which in turn have revealed the importance of this motif in catalysis [96–101]. Motif IV accommodates target adenine after it has flipped out of the DNA helix. The N^6 amino group forms hydrogen bonds to the side chain of serine or aspartic acid and one of the backbone oxygen atoms [21]. This would polarize N^6 negatively and activate it for direct transfer of the methyl group from AdoMet. In a random mutagenesis/selection study, Friedrich et al. [101] identified Asp^{193} (motif IV) in M.EcoRV as being a very important residue in catalysis, although the mutant D193G was able to bind DNA. Roth et al. [97] have constructed variants of M.EcoRV where aspartic acid of motif IV was replaced with either asparagine or alanine. Both of these variants were catalytically inactive, but were able to bind DNA and AdoMet with similar affinities as the wild-type M.EcoRV. Results with the D193N and D193A variants of M.EcoRV are in agreement with mutational data obtained with M.EcoKI, M.BcgI and EcoDam (DNA adenine MTase) [95,52,99]. In M.EcoKI, an NPPF to DPPF exchange resulted in a catalytically inactive enzyme that could still bind the AdoMet [95]. Variants of M.BcgI in which the NPPY asparagine residue was replaced by alanine, aspartic acid or glutamine were all catalytically inactive [52]. Motif IV has been proposed to be analogous to the catalytic PCQ motif of C^5-cytosine MTases. From the three-dimensional structures, it has been shown that the conserved NPPY motif in M.TaqI, an N^6-adenine MTase, overlaps with the PCQ motif of M.HhaI, a C^5-cytosine MTase, almost exactly when the two structures are superimposed [4,102,103]. In addition to aspartic acid, tyrosine is the second conserved amino acid residue in motif IV that has been investigated in detail. Pues et al. [49] have shown in the case of M.TaqI that replacement of Tyr^{108} with phenylalanine or tryptophan led to mutant MTases with almost identical or somewhat reduced enzymatic activities, compared with the wild-type M.TaqI. In contrast, the replacement of Tyr^{108} with alanine or glycine resulted in mutant MTases with dramatically reduced enzymatic activities which clearly demonstrates the importance of an aromatic amino acid side chain in motif IV for enzymatic activity. Similar results have been obtained with M.EcoRV [97] and M.EcoKI [95]. However, in the case of M.EcoP15I and M.EcoP1I, DPPY to DPPW exchange inactivated the enzyme [96,100].

In group γ, motif V contains the consensus sequence (N/D)LYXXF(L/V/I). The phenylalanine residue in motif V interacts through its aromatic side chain with the adenine of AdoMet. Schluckebier et al. [102] have shown that Leu^{142} (part of motif V) in M.TaqI makes van der Waals contacts with the adenine of AdoMet. It was therefore suggested in the case of groups α and β that one of the conserved hydrophobic side chains in motif V may have a same spatial position as the leucine residue in group γ MTases. Amino acids in motif VI constitute the C-terminal residues which are part of target adenine-binding site, whereas the arginine residue in motif VII is located in the putative DNA-recognition cleft possibly functioning in DNA binding. Ser^{229} of M.EcoRV, which is highly conserved in amino MTases, is located in motif VI and corresponds to Glu^{119} in M.HhaI. This residue is involved in acid–base catalysis in M.HhaI. Val-Phe, the consensus sequence of motif VIII forms the lower part of the target adenine-binding site and borders the DNA-binding cleft. The phenylalanine residue in this motif is well conserved (sometimes as tyrosine or tryptophan) in all amino MTases. This aromatic residue seems to make favourable contacts with the target DNA adenine in the case of M.TaqI. The high degree of conservation and the positioning of the side chain in the centre of the active site suggest that this aromatic amino acid holds a key role in catalysis [17]. In M.EcoRV, it has been shown clearly that Tyr^{258} (motif VIII) provides an aromatic ring and/or hydroxy group to interact with the adenine base, since the Y258A variant is catalytically almost inactive, but binds to DNA and AdoMet, although with reduced affinity for the AdoMet [97]. In M.TaqI, when Phe^{196} (motif VIII) was replaced with alanine, there was a significant decrease in the catalytic constant, whereas the F196W variant had almost wild-type catalytic activity, clearly indicating the importance of an aromatic amino acid in this position [49]. There is a marked difference in the position of motif X in the primary amino acid sequence of amino MTases and C^5-cytosine MTases. This motif is expected to form a helix next to the β-strand formed by motif I. Cheng [4] suggested that motif X, along with motif I and motif IV, provides the binding pocket for AdoMet's methionine moiety.

Structures

Although several DNA MTases have been sequenced and purified, only a handful of these MTases have been crystallized and their three-dimensional structures determined. M.HhaI [76], M.HaeIII [104] and human DNMT2 [105], belonging to the C^5-cytosine MTases, are the only three DNA MTases whose three-dimensional structures were solved in the presence of DNA. The three dimensional structures of six N^6-adenine MTases are known: M.TaqI [19,20], M.DpnM [106], M.RsrI [107,108], M.MboIIA [109], T4 Dam [110–112] and EcoDam [85].

In general, MTases are bilobal structures folded into two domains: a larger catalytic domain with both the active site for methyl transfer and the AdoMet-binding site and a smaller target (DNA)-recognition domain that possesses loops implicated in sequence-specific DNA recognition and the infiltration of the DNA to flip the target base. The DNA is bound in the cleft between the two domains flanked by several loops. The importance of some

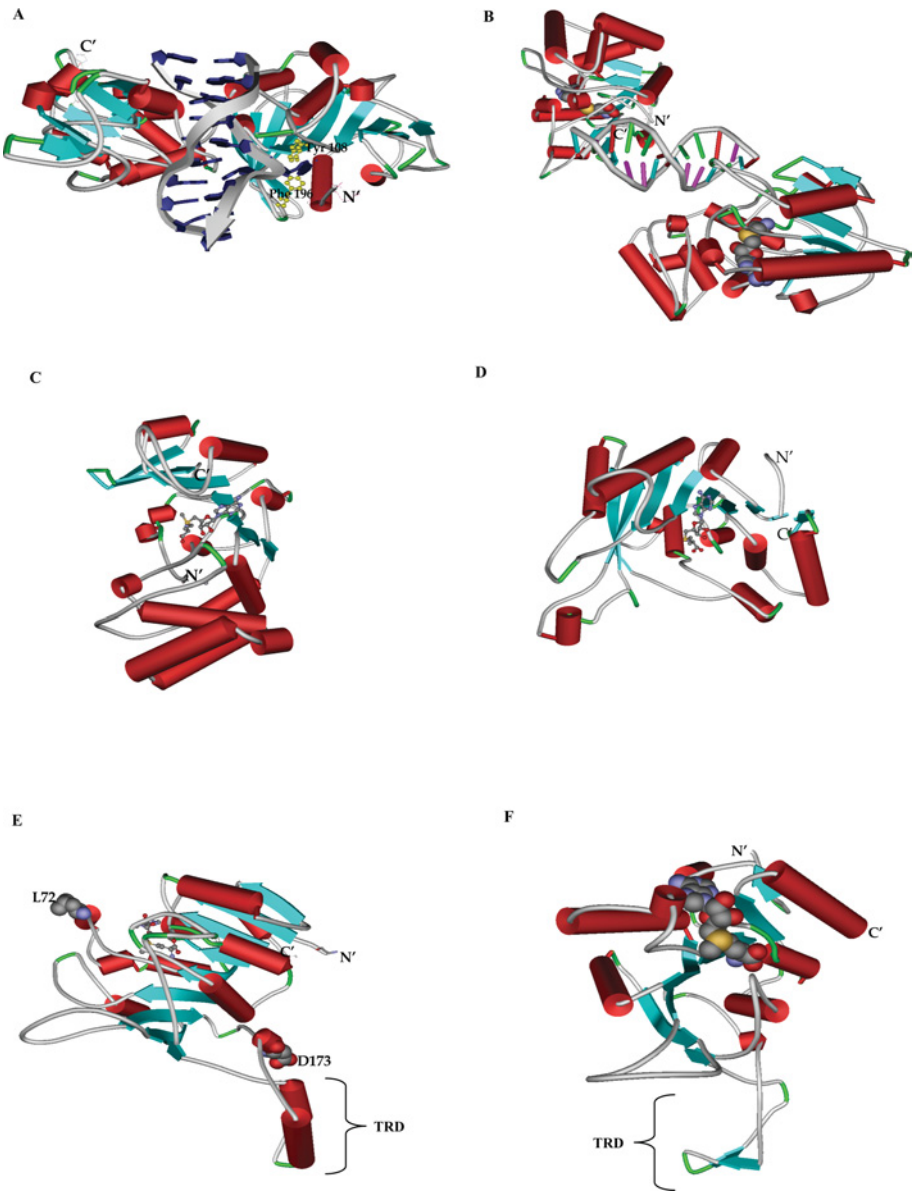

Figure 5 Structures of exocyclic DNA MTases

(**A**) Three-dimensional structure of M.TaqI with DNA (PDB code 1G38). The DNA backbone in the structure is shown as arrows and base pairs are shown as blue rings. The nucleotide shown in blue and present within the protein back bone is the flipped target adenine. Tyr^{108} and Phe^{196} shown in yellow are present in the vicinity of flipped target adenine. (**B**) The crystal structure of two molecules of T4 Dam bound to a palindromic 12 bp DNA duplex in presence of AdoHcy (PDB code 1Q0T). The DNA backbone in the structure is shown as tubes, base pairs are shown as sticks and AdoHcy molecules are shown as space-filled models. (**C**) The crystal structure of DpnM with AdoMet (PDB code 2DPM). The AdoMet molecule in the structure is shown as a ball and stick model. (**D**) Three-dimensional structure of M.PvuII in presence of AdoHcy (PDB code 1B00). The AdoHcy molecule in the structure is shown as a ball and stick representation. (**E**) The crystal structure of M.RsrI in presence of AdoMet (PDB code 1NW5). The AdoMet molecule in the structure is shown as a ball and stick model. The two amino acids (Leu^{72} and Asp^{173}) are represented as space-fill models. (**F**) The crystal structure of MboIIA in the presence of AdoMet (PDB code 1G60). The AdoMet molecule in the structure shown in space-fill representation.

amino acid residues located in these loops has been confirmed by several site-directed mutagenesis studies.

The large domain of all DNA MTases share a common structural core which consists of a six-stranded parallel β-sheet with a seventh strand inserted in an antiparallel fashion between the fifth and sixth strands (Figure 5). Within the large domain, one can clearly see two subdomains. One of the subdomains creates the AdoMet-binding site and the other subdomain is the binding site for the extrahelical target base. The large subdomain contains the nine characteristic amino acid motifs. The small domains of different DNA MTases are not similar in amino acid sequence, size or structure, and therefore cannot be superimposed.

All of these enzymes share a remarkably similar catalytic domain structure, resembling an α/β Rossman fold with conserved binding patterns for the cofactor AdoMet and modified base corresponding mainly to conserved motifs I and IV. In all cases, the substrate to be methylated is bound or expected to bind in a pocket adjacent to the AdoMet-binding site, which is formed by different amino acids in different MTases. Beyond the shared

catalytic MTase fold, the active sites of M.HhaI, M.TaqI, M.PvuII and M.RsrI are superimposable. The proposed catalytic function of motif IV in the amino MTases is to activate the exocyclic amine nucleophile by donating the hydrogen bonds from side chain (serine or aspartic acid) and backbone oxygen atoms [21]. The M.TaqI–DNA co-crystal structure confirms this proposal. The invariant DPPY of T4 Dam is superimposable on to the corresponding motif in the ternary complex of M.TaqI [112], as well as the binary complex of M.DpnII [106] and M.RsrI [107,108] in the absence of DNA, suggesting that the conformation of DPPY is quite stable and highly conserved.

Structural studies have provided evidence that the aspartic acid/aspargine/serine residue of motif IV [(D/N/S)PP(Y/F)] contacts the exocyclic amino group of the flipped base and activates it for methyl group transfer and the aromatic amino acid residue stacks to the flipped target base and stabilizes the flipped conformation. The structures of DNA MTases suggest that the flipped state is locked by structural rearrangements of the enzyme and DNA, which prevent back rotation of the flipped base.

The AdoMet-binding site is significantly conserved in all DNA MTases. In general, it is created by residues from the motifs I–III and X, which form conserved contacts to almost every hydrogen-bond donor and acceptor of AdoMet and, in addition, several hydrophobic interactions to the cofactor. The roles of many of these residues have been confirmed by mutagenesis studies. A V-shaped cleft in the molecule accommodates AdoMet in an extended conformation. In one arm of the V-shaped cleft, adenosine is located, while a sulfonium group ($S^{\delta+}$) occupies the tip of the cleft. The other arm harbours the methionine moiety. The positively charged amino acid residues in the loop region (connecting N- and C-terminal domains) are in position to interact with the phosphate groups of the DNA backbone. Interestingly, more than one AdoMet-binding site has been observed in M.PvuII [68], EcoDam [56,57] and T4 Dam [112,113]. The significance of the second AdoMet molecule is not clear. It has been suggested that the second AdoMet molecule (i) increases the occupancy of catalytically relevant AdoMet-binding site, and (ii) confers additional sequence specificity. Also, if the binding pocket for the methylatable base were occupied by the second AdoMet, base flipping would presumably require displacement of AdoMet and this could reduce the effective affinity for non-specific sites [68,83].

The structure of M.TaqI in complex with AdoMet, AdoHcy and with the competitive inhibitor sinefungin was determined at a resolution of 2.4 Å (1 Å = 0.1 nm) [19,20,66]. M.TaqI binds to adenosine moieties of AdoMet, AdoHcy and sinefungin in a similar manner, but the binding is different for their amino acid moieties [66]. The three-dimensional structure of M.TaqI with a specific 10 bp hemimethylated DNA and a non-reactive analogue 5′-[2(amino)ethylthio]-5′-deoxyadenosine showed that the target adenine present in the recognition sequence is indeed rotated out of the double helix to transfer the methyl group (Figure 5A) [20]. In the active site, two aromatic amino acid residues, Tyr^{108} (motif IV) and Phe^{196} (motif XIII) bind to the flipped target base, and the torsion of the methionine moiety of AdoMet by M.TaqI brings the methyl group within the reach of the flipped target base to transfer the methyl group. Both Tyr^{108} and Phe^{196} probably hold an important role in transfer of the methyl group by decreasing the energy of the positively charged transition state via cation–π interaction [17].

The ternary complex of T4 Dam with AdoHcy and 12 bp duplex revealed a non-specific loose DNA-binding mode with two Dam monomers bound to one duplex (Figure 5B). One T4 Dam binds to a single DNA duplex spanning 7 bp, whereas the other T4 Dam binds in the joint of two DNA molecules spanning 6 bp. There are no significant conformational changes in T4 Dam after non-specific binding in the ternary complex. However, the orientations of the two molecules of T4 Dam relative to the DNA helical axis are different. The first T4 Dam molecule is rotated by ~20 Å relative to the second T4 Dam molecule, resulting in a larger cavity for first one compared with the second T4 Dam [107–109]. Three structures for T4 Dam in ternary complexes with partially and fully specific DNA and a methyl donor analogue have been described. These structures illustrate the transition in enzyme–DNA interactions from non-specific to specific interactions, suggesting that there is a temporal order for formation of specific contacts. It is clear from such structural studies that T4 Dam moves along DNA and rotates up and down as a rigid body relative to the DNA. The ternary structure provides a rare snapshot of an enzyme poised for linear diffusion along the DNA.

The ternary structure of EcoDam in complex with cognate DNA is similar in many ways to the T4 Dam complex with cognate DNA. While the first base pair is contacted by Lys^{9} in EcoDam, Arg^{130} interacts with first guanine in T4 Dam. Interestingly, the flipped target adenine binds to the surface of EcoDam in the absence of AdoMet (a possible intermediate in the base flipping pathway) and the orphaned thymine displaying structural flexibility [85].

Among the exocyclic amino MTases, M.PvuII [21] is the only N^4-cytosine MTase whose three-dimensional structure is known (Figure 5D). Interestingly, in the structure of the M.PvuII–AdoMet complex, an extra electron density was found near the first AdoMet molecule. This density fitted for an adenosine moiety of AdoMet with the methionine moiety extending into the solvent.

Thomas et al. [108] determined the three-dimensional structures of M.RsrI in the presence of AdoMet, AdoHcy and sinefungin (Figure 5E). Two distinct binding conformations were observed for the three ligands. The substrate AdoMet adopts a bent shape that directs the activated methyl group towards the active site near the catalytic DPPY motif. The product AdoHcy and the competitive inhibitor sinefungin bind with a straight conformation in which the amino acid moiety occupies a position near the activated methyl group in the AdoMet complex. The two different modes of ligand binding by M.RsrI may be due to the differences in the positive charges on the ligands. The sulfur of AdoMet and the ε-amino group of sinefungin carry a formal positive charge, whereas AdoHcy is uncharged at the analogous position.

Szegedi and Gumport [54] observed that two mutants of M.RsrI (L72P and D173A) had altered catalytic and DNA-binding properties. The M.RsrI structure does provide an explanation for the functional defects of these mutants [107]. Leu^{72} is located in 3_{10}-helix D1, which is C-terminal to the catalytic motif, DPPY. Mutation of this residue to proline destabilizes helix formation of D1 or alters the flexibility of β-hairpin loop. This in turn results in the disruption of the normal conformation of the DPPY motif. The crystal structure of L72P was solved by Thomas et al. [108] and it showed that mutant M.RsrI did not co-crystallize with the cofactor. Substitution of Leu^{72} by proline caused a kink in the loop resulting in a compression of the loop on one side and the end of an α-helix shifted on the other side.

OLIGOMERIZATION STATUS AND BIOLOGICAL SIGNIFICANCE OF DIMERIC EXOCYCLIC AMINO MTases

In the cell, a DNA substrate for a typical MTase is hemimethylated and therefore needs only a single methylation event to convert it into a fully methylated state. This is in agreement with the fact that several of the DNA MTases studied to date exist as monomers in solution. Various studies, however, have revealed

an increasing number of apparently dimeric DNA MTases. For instance, M.BamHI [23] and chromosome-encoded adenine MTase CcrM [114] from *Caulobacter crescentus* occur as dimers in solution, but dissociate into monomers upon addition of the DNA. M.DpnII [115], M.EcoP15I [47,48,59,81,86], M.KpnI [35,84], M.LlaCI [116], M.MboIIA [109] and T4 Dam [112,113] have been shown to exist as dimers in solution. M.RsrI [117] has been shown to dimerize at high protein concentrations. Although the initial characterization of M.RsrI was consistent with the enzyme functioning as a monomer, it was shown later that the enzyme crystallized as a dimer. Re-examination of the biochemical properties of M.RsrI indicated that the M.RsrI–DNA complex had a size and stoichiometry consistent with a dimeric enzyme binding to DNA [118]. As with M.RsrI, M.MboIIA contains two tightly associated monomers having an interface area of $\sim$1900 Å^2 which is asymmetric in nature (Figure 5). The same residues in both monomers are involved in forming the interface. A three-dimensional model of M.KpnI based on the protein fold-recognition analysis, clearly predicted residues that are potentially important for the dimerization. Site-directed mutagenesis at the dimer interface drastically affected enzyme activity in addition to the oligomeric status of the enzyme. These results established that the monomeric MTase is catalytically inactive and dimerization is an essential event for M.KpnI to carry out the methyl-transfer reaction [84]. The intriguing similarity of the amino acid sequence of M.KpnI with that of dimeric Type III MTases, M.EcoP1I and M.EcoP15I [119], suggests their close evolutionary relationship. Monomeric Type II DNA MTases appear to have evolved from other lineages of the MTase superfamily, whereas M.KpnI has its origin in a dimeric Type III MTase that lost its Res subunit and acquired a Type II endonuclease partner from a different superfamily than the original nuclease subunit [120].

Sequence alignments reveal that the dimer interface amino acids may be conserved between M.RsrI, M.MboIIA and other dimeric β-class MTases. A motif $NXXTX_{9-11}AXRXFSXXHX_4$-$WX_{6-9}YXFXLX_3RX_{9-26}NPX_{1-6}NVWX_{29-34}A$ is thought to be defined as the dimerization interface [118]. The increasing number of dimeric MTases raises a question as to whether dimerization is important for catalysis. It is possible that dimerization stabilizes and enhances site-specific DNA binding by the MTase, because the DNA-binding site may require an interfacial structure formed by both monomers. The dimeric form of a MTase allows the formation of a large enzyme–substrate network with high-molecular-mass DNA. As first proposed by Dong et al. [121], dimeric MTases might provide an evolutionary link among different types of R-M systems. Type IIH MTases form a clear link between Type I and II R-M systems by utilizing gene fusions and the shuffling of structural and functional elements [103].

It has been proposed that N^6-adenine MTases and N^4-cytosine MTases, which closely resemble one another, derive from a common ancestor [1]. At least four N^6-adenine MTases have been shown to methylate cytosine residues at position N^4, although at reduced rates [5,122], and therefore postulated to belong to one family of exocyclic amino MTases. However, a phylogenetic analysis of MTases suggests that these MTases are found on distinct branches of a tree, suggesting a very ancient divergence of both subfamilies of exocyclic MTases [123].

FUTURE PERSPECTIVES

There are several interesting open questions regarding exocyclic amino MTases. Although some of the adenine DNA MTases follow an ordered mechanism and others follow a random mechanism, it would be interesting to see how these mechanistic differences are reflected in the structures of the corresponding enzyme–DNA complexes. There are significant differences in the $k_{methylation}$ and k_{cat} values between the exocyclic (N^4-cytosine and N^6-adenine) and ring (C^5-cytosine) MTases. It is not clear at this point in time what determines this difference. Is methyl group transfer to an exocyclic amino group compared with a ring cytosine the determining factor, or is it the nature of the target base? What properties of the N^4-cytosine MTases correlate with the N^6-adenine MTases and how are these MTases related to the type of chemistry they perform? In spite of much progress, two key questions with respect to the mechanism of base flipping are unresolved. It is not known whether the MTases actively initiate the rotation of the base or whether they rely on breathing of DNA and only capture the flipped base after the rotation has occurred passively. Secondly, the temporal coupling of DNA recognition and base flipping is not clearly understood, in particular because no structure of a DNA MTase with an unflipped DNA is available. The key question is whether DNA recognition starts before or after base flipping. Although the replication fork moves at 1000 bp/s and on average produces four Dam sites/s, the rate of Dam methylation by EcoDam is 6 s^{-1}. Future work should focus on possible mechanical coupling of Dam and DNA replication. More structural work will be required to understand why some MTases are processive in their mode of action, whereas others are distributive in nature. The available sequence information on exocyclic MTases needs to be complemented by enzyme–DNA structures of related enzymes with different recognition sites. Such studies will help us to understand the molecular evolution of DNA recognition. Dam methylation is essential for the virulence of a growing list of bacterial pathogens. Adenine MTases are not present in mammals and, considering the fact that adenine methylation is crucial for bacteria, these enzymes could be interesting drug targets.

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